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Noncovalent Interaction of Lactoferrin with Epicatechin and Epigallocatechin: Focus on Fluorescence Quenching and Antioxidant Properties

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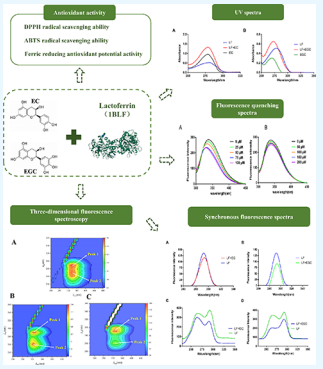
ABSTRACT: Lactoferrin (LF) from bovine milk possesses antioxidant activity, immune regulatory and other biological activities. However, the effects of epicatechin (EC) and epigallocatechin (EGC) interacting with LF on the antioxidant activity of LF have not been investigated. Therefore, this study aimed to explore their interaction mechanism and the antioxidant activity of LF. UV spectra revealed that EGC (100 μM) induced a higher blue shift of LF at the maximum absorption wavelength than that of EC (100 μM). Fluorescence spectra results suggested that LF fluorescence was quenched by EC and EGC in the static type, which changed the polarity of the microenvironment around LF. The quenching constants K_{sv} (5.91×10^3−9.20×10^3) of EC-LF complexes at different temperatures were all higher than that (1.35×10^3−1.75×10^3) of the EGC-LF complex. EC could bind to LF via hydrophobic interactions while hydrogen bonding and van der Waals forces drove the binding of EGC to LF. Both the EC-LF complex and EGC-LF complex could bind to LF with one site. EGC formed more hydrogen bonds with LF than that of EC. The antioxidant activity of LF was increased by the high addition level of EC and EGC. These findings would provide more references for developing LF-catechin complexes as functional antioxidants.



1. INTRODUCTION

Polyphenols in tea have been widely proven to exert antioxidant, anti-inflammatory, and anticancer activity,[1−3] which can be divided into catechin, flavones, and other phenolic acids. Catechin represents the major variety of the polyphenols existing in tea, which mainly contains epicatechin (EC), epigallocatechin (EGC), epicatechin-3-gallate (ECG), and epigallocatechin-3-gallate (EGCG).[4] The antioxidant activity of catechins relies on hydroxyl groups and regulating related cell signaling pathways, which contributes to its anti-inflammation, anticancer, and the ability to alleviate cardiovascular disease. Nowadays, fabricating protein−polyphenol complexes has been selected as an effective strategy to improve the antioxidant activity and stability of proteins.[5] In previous studies, the antioxidant activity of EGCG and EGCG-protein complexes has attracted more attention than that of other kinds of catechins. EC and EGC also could display antioxidant activity and inhibit proliferation of cancer cell lines to benefit human health.[6] However, studies on the effects of EC and EGC interacting with LF on its antioxidant activity have been scarcely available.

Protein can interact with polyphenols to form protein−polyphenol complexes via noncovalent bonds, which changes the structural and functional properties of protein and polyphenols. Due to the catechol structure of polyphenols, the binding of polyphenols with protein makes polyphenol−protein complexes exert higher antioxidant activity. Previous studies showed that rice glutelin increased both 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability and ferric reducing antioxidant potential (FRAP) of proanthocyanidin B2 during storage.[7] Zhang et al. reported that the noncovalent interaction between pea protein isolate (PPI) and catechin enhanced DPPH radical and ABTS radical scavenging capacities of the PPI−catechin complex.[8] Moreover, the interaction of polyphenols with protein from bovine milk has been extensively reported in recent years. Wang et al. found that the antioxidant activity of α-lactalbumin alone was lower than the α-lactalbumin-EGCG complex.[9] Cyanidin-3-*O*-glucoside (C3G) was also used to enhance the radical scavenging activity, reducing power and Fe^{2+} chelating capacity of whey protein isolate (WPI) and β-lactoglobulin (β-LG)[9] by forming C3G-protein complexes. Moreover, covalent modification of β-LG by EGCG possessed higher scavenging DPPH radical activity and FRAP than β-LG.[10] These studies implied that protein−polyphenol complexes could enhance the antioxidant activity

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of protein, which further increased the nutrition value of food enriched with protein and polyphenols.

Lactiferrin (LF) is known from various species,[11−15] and bovine LF with a molecular weight of around 80 kDa is an iron-binding glycoprotein from the whey fraction of cow's milk.[16,17] Due to the existence of tryptophan in LF, the changes in the fluorescence intensity of tryptophan residue at 295 nm reflect the tertiary structural changes of LF.[18] Regular intake of LF has various health benefits, such as anti-inflammatory, antimicrobial, immune regulatory, and antitumor activity. Therefore, LF accompanied by polyphenols has been widely applied in functional products.[19] However, most studies have focused on the effects of the interaction between LF and EGCG,[20,21] tannic acid,[22] dihydromyricetin and myricetin[23] on the physicochemical properties of LF, and the effects of the interaction between EC and EGC with LF on the antioxidant activity of their complexes have not been reported.

In this study, the interaction mechanism of EC and EGC with LF on the antioxidant activity of LF could be revealed via fluorescence spectroscopy. Moreover, molecular docking was also adopted to analyze the binding sites of EC/EGC and LF. This study will provide evidences for the application of catechin in functional food enriched with LF.

2. MATERIALS AND METHODS

2.1. Materials. LF (purity >98%) was ordered from Hilmar Ingredients (Dalhart, TX, USA). Potassium persulfate, DPPH, ABTS, and 2,4,6-tripyridyl-s-triazine (TPTZ) were purchased from Shanghai Yuanye Biotechnology Co., Ltd. EGC and EC were obtained from Biopurify Phytochemicals Ltd. (Sichuan, China), which were both of HPLC grade. All other reagents were of analytical grade.

2.2. LF-Catechin Complex Preparation. LF was dispersed in phosphate buffer (20 mM, pH 7) to obtain 4 and 0.02 mg/mL LF solution. The above LF solution was used to disperse different amounts of EC and EGC to make LF-catechin complexes with different LF/catechin ratios, according to the method that Meng et al. reported.[24] The LF-catechin complexes were further used for the analysis of the fluorescence spectra and antioxidant activity.

2.3. UV Spectra Analysis. UV spectra can be adopted to analyze the phenomenon of red-shift and blue-shift occurring in proteins due to the interaction between protein and polyphenols.[23] The solutions of LF with 0.04 and 0.02 mg/mL, EC (100 μM), EGC (100 μM), and LF-catechin complexes were prepared. The UV absorption spectra of LF, EC, EGC, and LF-catechin complexes were scanned in the range of 200−400 nm with a wavelength interval of 1 nm.

2.4. Fluorescence Quenching Analysis. Fluorescence spectra were used to analyze the interacting mechanism of LF with EC and EGC by a Cary Eclipse fluorescence spectrophotometer (Agilent, USA). The types of fluorescence quenching contained static quenching and dynamic quenching.[25] The LF concentration was fixed at 4 mg/mL, and the LF/catechin complexes with different LF/catechin ratios were prepared with final concentrations of EC (50, 100, 150, and 200 μM) and EGC (0, 25, 50, 75, and 100 μM) in LF solution (4 mg/mL). The effect of different concentrations of EC and EGC on LF fluorescence at 298, 308, and 318 K was determined at excitation wavelength of 200 nm and emission wavelength range of 200−500 nm. The slit widths of excitation and emission were both set at 5.0 nm with a voltage of 600 V.

The Stern−Volmer eq 1 can be employed to analyze the mechanism of EC and EGC on quenching LF fluorescence.[26]

$$\frac{F_0}{F} = 1 + K_{SV}[Q] = 1 + K_q\tau_0[Q] \quad (1)$$

Here, F_0 and F represent the fluorescence intensity of LF and LF-catechin complexes, respectively, and Q represents the concentration of EC and EGC. K_q is the quenching rate constant of the biomolecule. K_{sv} is the Stern−Volmer quenching constant. τ_0 is the average lifetime of the fluorophore without quenchers, and the constant value is around 1×10^{-8} s.

The binding constant (K_A) and number of binding site (n) can be calculated by eq 2.[27]

$$\lg\frac{F_0 - F}{F} = \lg K_A + n\lg[Q] \quad (2)$$

where Q is the concentrations of EC and EGC and F_0 and F are the fluorescence intensities of LF and LF-catechin complexes, respectively.

2.4.1. Thermodynamic Analysis. According to eqs 3 and 4,[28] the thermodynamic parameters (ΔH, ΔS,p and ΔG) are adopted to analyze the interaction forces between catechin and LF.

$$\ln K_a = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad (3)$$

$$\Delta G = \Delta H - T\Delta S \quad (4)$$

where the temperature T was set at 298, 304, and 310 K and R is the gas constant 8.314 J/(mol K). ΔS, ΔH, and ΔG represent the change of entropy, enthalpy, and the free energy, respectively.

2.4.2. Synchronous Fluorescence Spectra analysis. A Cary Eclipse fluorescence spectrophotometer (Agilent, USA) was used to investigate the changes of the microenvironment near tryptophan and tyrosine of LF caused by EC and EGC. The concentration of LF was kept at 2 mg/mL, and the synchronous fluorescence spectra of LF mixed with EC (100 μM) and EGC (100 μM) solutions were measured at λ_{em} interval (15 nm) and λ_{ex} interval (60 nm).

2.4.3. Three-dimensional fluorescence spectroscopy analysis. Three-dimensional fluorescence spectroscopy can also be used to analyze the interaction between LF and catechin. The three-dimensional spectra of LF (2 mg/mL), LF-EC (100 μM) complex, and LF-EGC (100 μM) complex were scanned at an excitation wavelength of 200 nm and emission wavelength range of 200−500 nm. The emission and excitation slit were both set at 5 nm, and the scan speed was set at 600 nm/min, with the voltage of 600 V and the gain value of 10.

2.5. Molecular Docking Analysis. The 3D crystal structure file of LF was downloaded from the PDB (ID: 1BLF) database.[29] The water molecules and other heteroatoms in the crystal structure were deleted, and the metal ions were retained. The molecular structure of EC and EGC was downloaded from the Pubchem database.[29] Chemoffice software was used for minimizing energy in the MMF94 force field.[30] The processed protein receptor and small molecule ligand files were converted to .pdpqt format using Autodock Tools.[30] Autodock vina was used to explore the binding sites between LF and EC/EGC. The point parameters of docking grid were as follows: spacing = 1, center_x = 50.306, center_y = 60.166, center_z = 22.764, size_x= 88, size_y = 66, size_z = 58. The docking box was made to cover the whole protein receptor, and blind docking was

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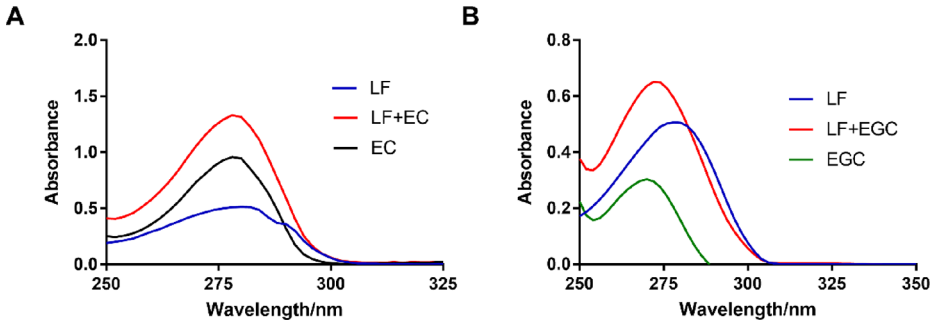

Figure 1. UV spectra of the catechin and catechin-LF complexes. (A) UV spectra of LF, EC, and LF-EC complexes. (B) UV spectra of LF, EGC, and LF-EGC complexes.

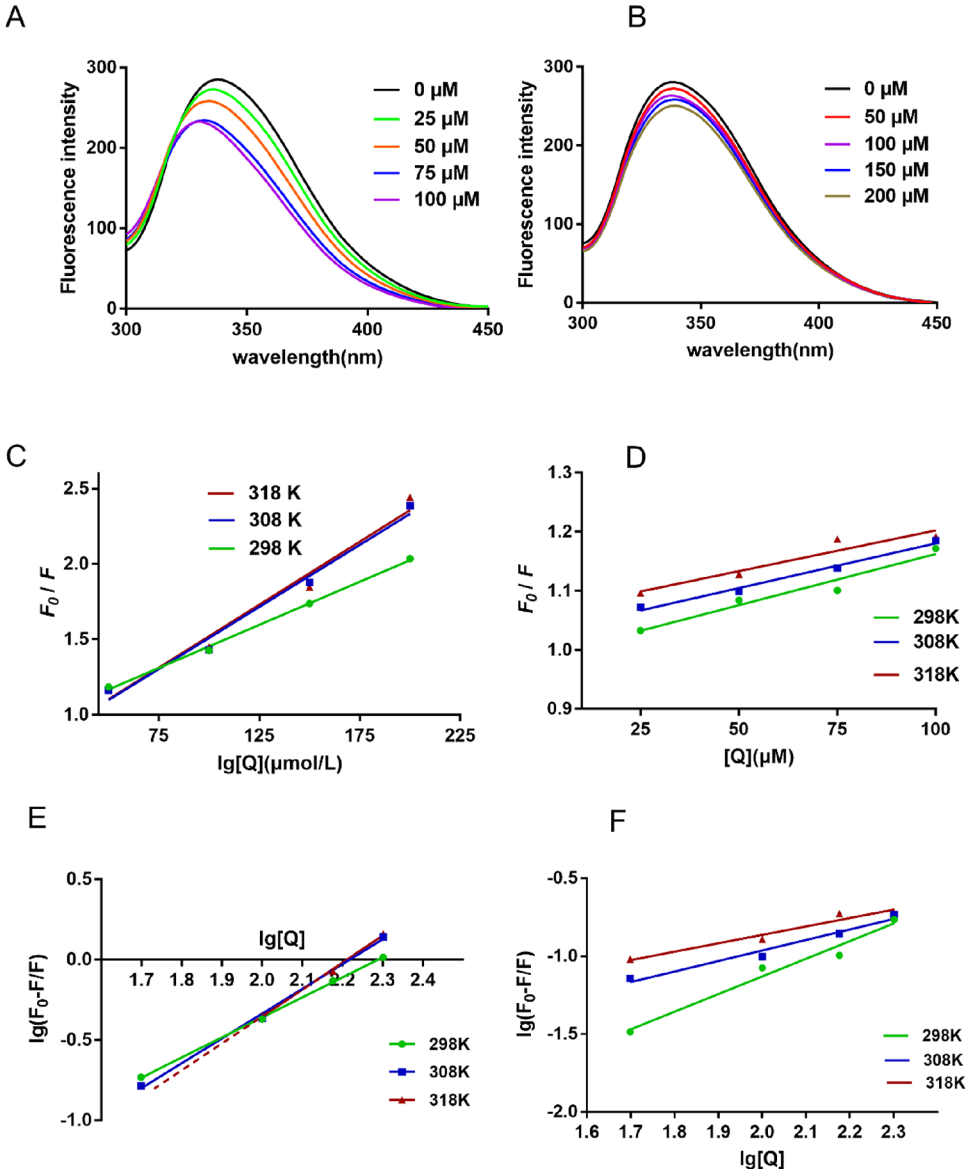

Figure 2. Fluorescence spectra, Stern−Volmer plots, and double logarithmic curves for the quenching of LF fluorescence in the presence of EC and EGC. Fluorescence quenching spectra of LF in the presence of EC (A) and EGC (B). Stern−Volmer plots for the quenching of LF by EC (C) and EGC (D) at 298, 308, and 318 K. Double logarithmic curves of interaction between EC/EGC and LF at 298, 308, and 318 K.

performed to seek potential sites of LF binding to ligands. Pymol software and Discovery studio 2019[31] were used to analyze the docking results.

2.6. Antioxidant Activity. *2.6.1. DPPH Radical Scavenging Activity.* The DPPH scavenging radical activity of catechin and LF-catechin complexes was determined by the method that Barreira et al. described[32] with some modifications. LF solution

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Table 1. Quenching Constants of Interaction between EC/EGC and LF at Different Temperatures

	EC			EGC		
temperature/K	K_{sv}(10^3 L/mol)	K_q(10^{11} L/mol·s)	R^2	K_{sv} /(×10^3 L/mol)	K_q /(×10^{11} L/mol·s)	R^2
298	5.91 ± 0.03	5.91 ± 0.03	0.9998	1.35 ± 0.07	1.35 ± 0.07	0.9526
308	8.90 ± 0.10	8.90 ± 0.10	0.9984	1.50 ± 0.00	1.50 ± 0.00	0.9884
318	9.20 ± 0.11	9.20 ± 0.11	0.9871	1.75 ± 0.07	1.75 ± 0.07	0.9155

(2 mg/mL) was chosen as the solvent to prepare LF-EC complex solution with different EC concentrations (25, 50, and 100 μM) and LF-EGC complex solution with different EGC concentrations (15, 30, and 60 μM). The solutions of EC, EGC, LF-EC, and LF-EGC complex solutions (100 μL) were mixed with 900 μL of DPPH solution (0.1 mM) in the dark for 20 min, respectively. The absorbance of the reaction solution was measured at 517 nm. The ethanol in replace of sample solution to react with DPPH solution was taken as control. The DPPH scavenging radical activity was calculated according to eq 5,

$$\text{DPPH radical scavenging activity}(\%) = \left(1 - \frac{A_{\text{sample}}}{A_{\text{control}}}\right) \times 100 \quad (5)$$

where A_{sample} represents the absorbance of catechin and catechin-LF complex solution reacting with DPPH solution and A_{control} is the absorbance of control with DPPH solution.

2.6.2. ABTS Radical Scavenging Activity. According to the method that Yuan et al. reported,[33] the DPPH scavenging radical activity of catechin and LF-catechin complexes was measured. LF-EC and LF-EGC complex solutions with different LF/catechin ratios were prepared with the final EC concentration of 7.5, 15, and 30 μM, and final EGC concentrations of 5, 10, and 20 μM. The LF solution was fixed at 2 mg/mL. The catechin and LF-catechin complex solutions (100 μL) with different LF/catechin ratios reacted with ABTS solution (900 μL) for 30 min. The absorbance of the above solutions was determined to be 734 nm. The ethanol in replace of sample solution to react with ABTS solution was taken as control. The ABTS scavenging activity was calculated according to eq 6,

$$\text{ABTS radical scavenging activity}(\%) = \left(1 - \frac{A_{\text{sample}}}{A_{\text{control}}}\right) \times 100 \quad (6)$$

where A_{sample} represents the absorbance of catechin and LF-catechin complex solution reacted with ABTS solution and A_{control} is the absorbance of control with ABTS solution.

2.6.3. FRAP Assay. The FRAP activity of catechin and LF-catechin complexes was measured according to the assay that Zhang et al. reported.[34] LF solution (2 mg/mL) and LF-EC and LF-EGC complex solutions with different LF/catechin ratios were prepared with final EC concentrations of 15, 30, and 60 μM and final EGC concentrations of 10, 20, and 40 μM, respectively. The reaction of catechin and LF-catechin complex solutions mixed with FRAP solution was kept in the dark. After 30 min, the absorbance of the reaction solution was measured at 593 nm. The FRAP value of different samples was expressed as the absorbance value.

3. RESULTS AND DISCUSSION

3.1. UV Spectra of LF with EC and EGC. The changes of the UV absorption peaks of protein indicate changes in the microenvironment around amino acid residues of protein. The UV spectra of catechin and catechin-LF complexes are shown in Figure 1. It can be seen that the maximum absorption peak of LF appeared at 280 nm due to the presence of tryptophan, tyrosine, and phenylalanine. LF-EC complex had a maximum absorption peak at 278 nm with a blue shift (from 280 to 278 nm), compared with LF. EGC displayed a maximum absorption peak at 270 nm, and the addition of EGC caused a blue shift (from 280 to 272 nm) at the maximum absorption peak of LF, which suggested that both EC and EGC changed the polarity of LF. Similar results were also found in the maximum absorption peak of LF interacting with myricetin.[23] Compared with EC (100 μM), EGC (100 μM) caused a higher blue shift of LF at the maximum absorption wavelength. The above results implied that EGC might cause higher changes of LF structure than EC due to the discrepancy of their hydroxyl number.

Table 2. Binding Constants and Binding Sites of EC/EGC with LF at 298, 308, and 318 K

	EC		EGC	
temperature/K	K_A/(10^8 L/mol)	n	K_A/(10^8 L/mol)	n
298	7.16	1.25 ± 0.01	26.37 ± 2.17	1.14 ± 0.01
308	26.32	1.54 ± 0.01	1.97 ± 0.12	0.67 ± 0.02
318	50.95	1.68 ± 0.02	0.85 ± 0.04	0.54 ± 0.01

3.2. Fluorescence Spectroscopy Analysis of LF-EC and LF-EGC Complexes. Fluorescence spectroscopy is a conventional technology used for studying the interaction mechanism of polyphenol binding with protein. Since the tyrosine and tryptophan of LF could produce endogenous fluorescence, LF could emit fluorescence at a constant excitation wavelength. In Figure 2A,B, LF exhibited a maximum emission wavelength (340 nm) at an excitation wavelength of 295 nm due to the tryptophan residues.[35] The fluorescence intensity of LF without catechin was the highest and decreased with the increasing concentrations of EC (50−200 μM) and EGC (25−100 μM), which showed a dose−effect relationship. These results indicated that the binding of catechin and LF led to the quenching of LF fluorescence. Huang et al. also reported that tannic acid could decrease the fluorescence intensity of LF due to the formation of tannic acid-LF.[23] The effect of EGC (100 μM) on the quenching fluorescence intensity of LF was stronger than that of EC (100 μM). These results suggested that both EC and EGC directly interact with the amino acid residues of LF.

3.3. Mechanism of LF Fluorescence Quenched by EC and EGC. The Stern−Volmer equation was used to analyze the mechanism of LF fluorescence quenched by EC and EGC. Static quenching and dynamic quenching are the common types for the quenching mechanism. Static quenching occurs in the formation of a complex between fluorophore and quencher, while dynamic quenching resulted from diffusion and collision encounters. As shown in Figure 2C,D, the Stern−Volmer plots for the EC-LF complex and EGC-LF complex at different temperatures showed linear (R^2 > 0.97), suggesting that both EC and EGC quenched LF fluorescence in dynamic type.[8] The

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Table 3. Thermodynamic Parameters of the Interaction between EC/EGC and LF at Different Temperatures

	EC			EGC		
T (K)	ΔH (kJ/mol)	ΔG (kJ mol^{-1} K^{-1})	ΔS (kJ mol^{-1} K^{-1})	ΔH (kJ/mol)	ΔG (kJ/mol)	ΔS (kJ mol^{-1} K^{-1})
298	129.43 ± 0.52	−0.22 ± 0.01	0.44 ± 0.01	−135.90 ± 5.05	−18.86 ± 0.14	−0.39 ± 0.02
308		−4.57 ± 0.01			−14.93 ± 0.02	
318		−8.92 ± 0.03			−11.00 ± 0.19	

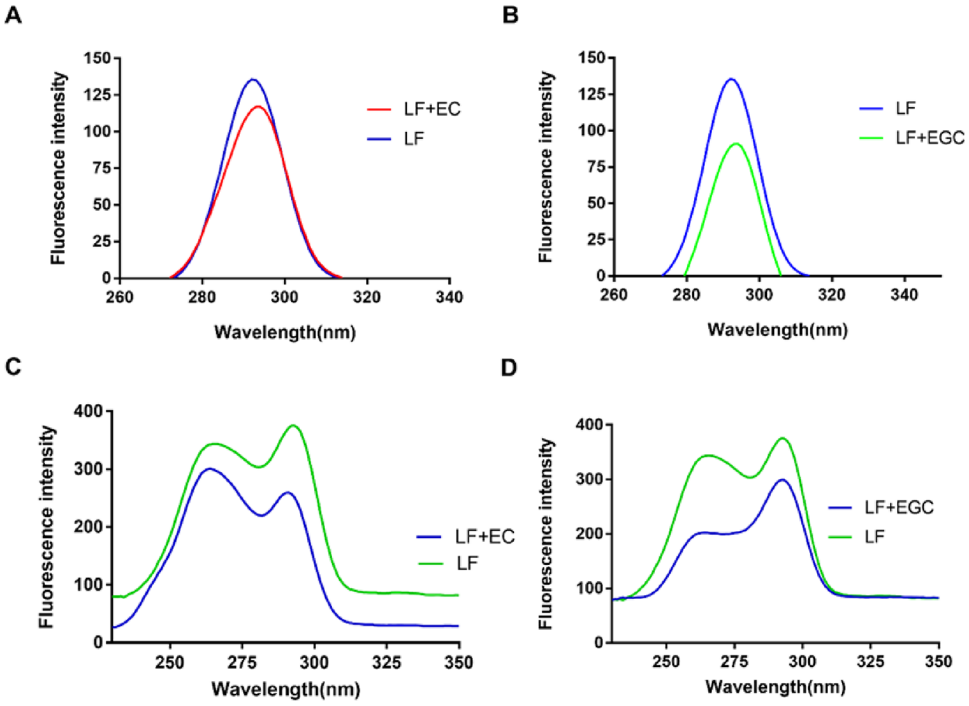

Figure 3. Synchronous fluorescence spectra of LF quenched by EC and EGC at $\Delta\lambda$ = 15 and 60 nm. (A) Synchronous fluorescence spectra of LF in the absence of EC at $\Delta\lambda$ = 15 nm. (B) Synchronous fluorescence spectra of LF in the absence of EGC at $\Delta\lambda$ = 15 nm. (C) Synchronous fluorescence spectra of LF in the absence of EC at $\Delta\lambda$ = 60 nm. (D) Synchronous fluorescence spectra of LF in the absence of EGC at $\Delta\lambda$ = 60 nm.

values of K_{SV} and K_q for the LF-catechin complex are listed in Table 1. The quenching constant K_{SV} of the EC-LF complex and EGC-LF complex increased with the increasing temperature. The K_{SV} values for the EC-LF complex at 298, 308, and 318 K were 5.91 × 10^3, 8.90 × 10^3, and 9.20 × 10^3, respectively. The K_{SV} values for the EGC-LF complex at different temperatures ranged from 1.35 × 10^3 to 1.75 × 10^3, which were lower than that of the EC-LF complex. The K_q for both the EC-LF complex and EGC-LF complex also increased with the increasing temperature. The K_q values for the EC-LF complex were 5.9 × 10^{12} (298 K), 8.9 × 10^{12} (308 K), and 9.2 × 10^{12} (318 K), and the K_q for the EC-LF complex at different temperatures was higher than that of EGC-LF. Moreover, the K_q for both the EC-LF complex and EGC-LF complex was higher than the limiting diffusion collision quenching constant of 2.0 × 10^{10} L/(mol·s).

3.4. Binding Constants (K_A) and Binding Sites (n) of LF and EC/EGC. The double-logarithmic plots of the EC-LF complex and EGC-LF complex are shown in Figure 2E,F, and the values of K_A and n are shown in Table 2. The K_A values of EC binding to LF increased with increasing temperature, while the K_A of the EGC-LF complexes decreased with rising temperature. The K_A values for the EC-LF complex were 7.16 × 10^8 (298 K), 26.32 × 10^8 (308 K), and 50.95 × 10^8 (318 K). The results suggested that the interaction between EC and LF became stronger with higher temperature, and the EC-LF complex became stable at higher temperature. Due to more hydroxyl in EGC, the EGC-LF complex displayed the contrary tendency. The K_A values for the EGC-LF complexes at 298, 308, and 318 K were 26.37 × 10^8, 1.97 × 10^8 and 0.85 × 10^8, respectively. The stability of the EGC-LF complex could decrease at high temperature. The K_A values for the EC-LF complex and EGC-LF complex were both in the level of 10^8, which were higher than the binding constant of the lentil protein isolate-C3G complex[36] and C3G-β-LG complex.[37] The binding site number of EC/EGC binding to LF at three different temperatures was both around one, suggesting that both EC and EGC have one binding site on LF.

3.5. Thermodynamic Parameters and Binding Forces of the Interaction between LF and EC/EGC. The binding forces between protein and quenchers mainly contain several noncovalent forces such as electrostatic forces, hydrophobic interactions, hydrogen bonds, and van der Waals forces. The interaction forces between catechin and LF can be determined by the thermodynamic parameters of ΔH, ΔS, and ΔG. Hydrophobic interactions are the vital forces when $\Delta H > 0$ and $\Delta S > 0$. $\Delta H < 0$ and $\Delta S < 0$ mean hydrogen bonding and van der Waals forces. Electrostatic interactions play the main role in the binding of protein and quenchers when $\Delta H < 0$ and $\Delta S > 0$.[38]

The thermodynamic parameters of binding of EC and EGC to LF are shown in Table 3. The ΔG values of the EC-LF complex and EGC-LF complex were both negative, indicating that the

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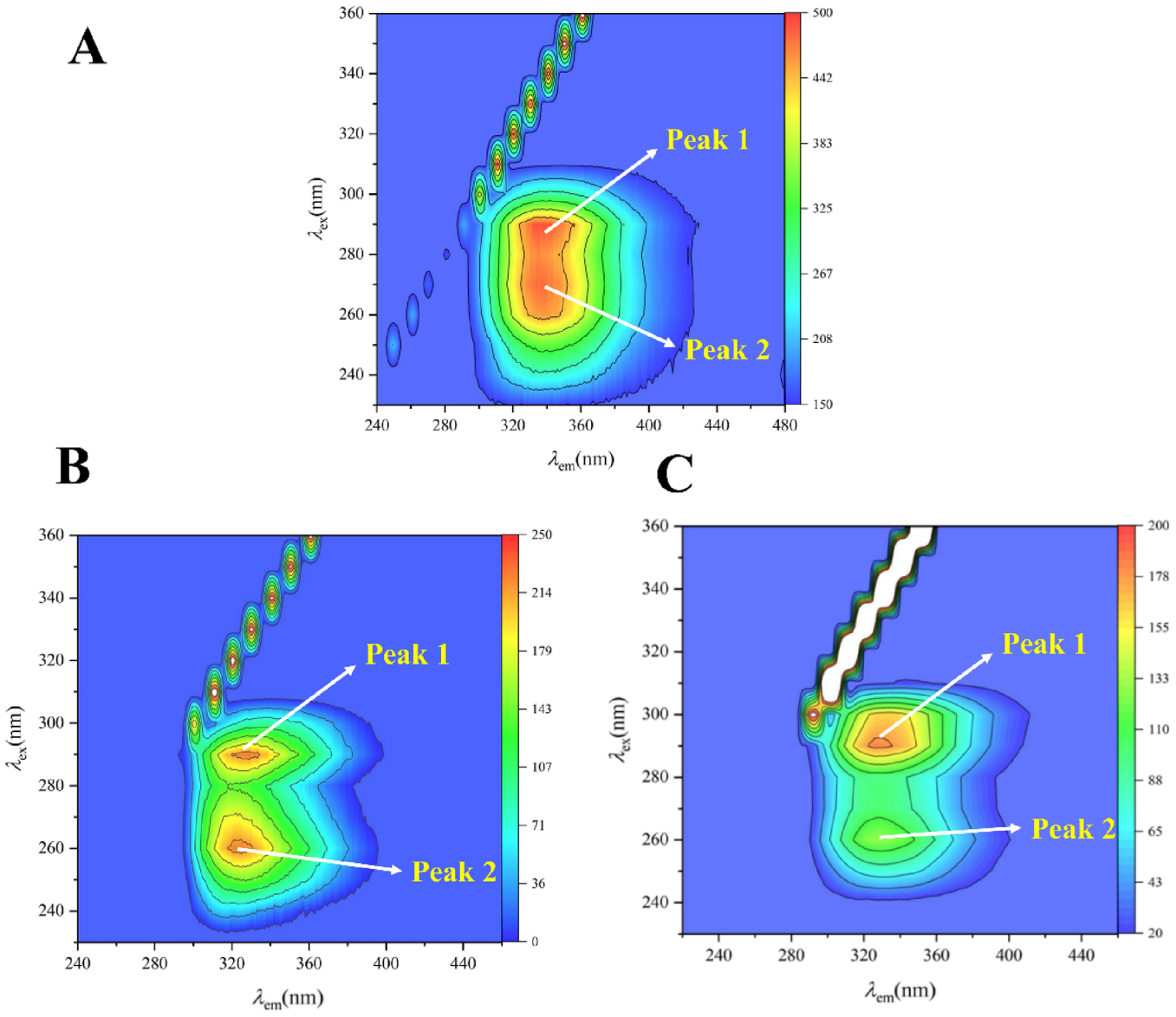

Figure 4. 3D fluorescence spectroscopy of LF and the LF-catechin complex. A: LF, B: LF-EC complex, C:LF-EGC complex.

binding of EC and EGC to LF both occurred in a spontaneous type. For the EC-LF complex, ΔH (129.43 kJ/mol) > 0 and ΔS (0.44 kJ mol^{-1} K^{-1}) > 0 indicated that hydrophobic interactions dominated the binding of EC to LF. A similar phenomenon was also found in the interaction between SPI and catechin.[39] The ΔH (−135.90 kJ/mol) 0 and ΔS (−0.39 kJ mol^{-1} K^{-1}) 0 suggested that the formation of the EGC-LF complex was driven by hydrogen bonding and van der Waals forces. The discrepancy on the binding forces of EC-LF complex and EGC-LF complex might be attributed to the different numbers of hydroxyl on their molecule structure.

3.6. Synchronous Fluorescence Spectra of LF, LF-EC, and LF-EGC Complexes. The synchronous fluorescence spectra of protein and protein−catechin complexes could reflect the microenvironmental change of tyrosine and tryptophan residues in protein. The change of the maximum emission wavelength represents the polarity change of LF. In Figure 3, the fluorescence peak of tyrosine residues in LF appeared at 292 nm wavelength.[40] Both the EC-LF complex and EGC-LF complex had caused a red shift from 292 to 294 nm at $\Delta\lambda$ = 15 nm, which suggested that both EC and EGC lowered the hydrophobicity around the tyrosine residues of LF. Both EC and EGC induced a blue shift of the tryptophan residue peak from 266 to 264 nm, indicating that EC and EGC could increase higher hydrophobicity around the tryptophan residues. Therefore, the interaction between LF and catechin led to microenvironmental change of tyrosine and tryptophan residues in LF, which further verified that the tertiary structure of LF was altered by EC and EGC.

3.7. Three-Dimensional Fluorescence Spectroscopy Analysis of LF, LF-EC, and LF-EGC Complexes. Three-dimensional fluorescence spectra can be used to reveal the conformation changes of LF induced by EC and EGC. In Figure 4A, LF alone displayed two peaks in three-dimensional fluorescence spectra including peak 1 ($\lambda_{ex}/\lambda_{em}$ = 290/335 nm) and peak 2 ($\lambda_{ex}/\lambda_{em}$ = 270/337 nm), which were generated by the tyrosine and tryptophan residues of LF. The addition of EC and EGC did not cause the change of peak 1 position. However, both EC and EGC caused a significant decrease in the fluorescence intensity of peak 1. Moreover, the addition of EC

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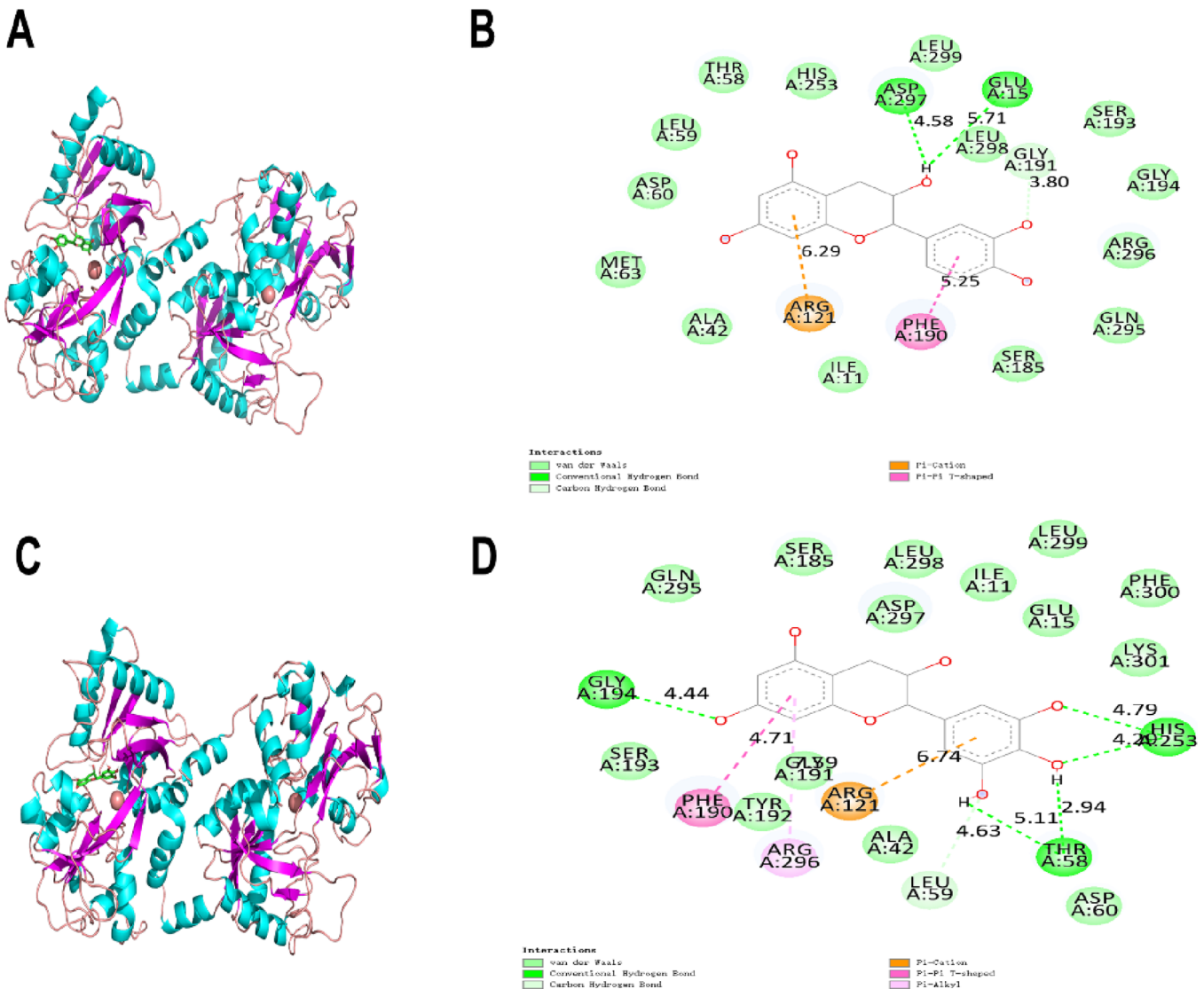

Figure 5. 3D view of LF interacting with EC (A) and EGC (C). 2D schematic interaction diagram of LF and EC (B) and EGC (D). Hydrogen bonds were expressed as the green short-dash lines.

Table 4. Distance between EC/EGC and the Amino Acid Residues of LF

EC		EGC	
interacting amino acids of BLF	distance between EC and the amino acid residue	interacting amino acids of BLF	distance between EGC and the amino acid residue
Asp297 (hydrogen bond)	4.58 Å	Gly194 (hydrogen bond)	4.44 Å
Glu15 (hydrogen bond)	5.71 Å	His253 (hydrogen bond)	4.79 Å/4.29 Å
Arg121 (π-cation)	6.29 Å	Thr58 (hydrogen bond)	2.94 Å/4.63 Å
Phe190 ($\pi-\pi$)	5.25 Å	Arg296 (π-alkyl)	7.59 Å
		Phe190 ($\pi-\pi$)	4.71 Å
		Leu59 (carbon hydrogen bond)	4.63 Å

and EGC made the position of peak 2 ($\lambda_{ex}/\lambda_{em}$ = 270/337 nm) shift to $\lambda_{ex}/\lambda_{em}$ = 260/325 nm and $\lambda_{ex}/\lambda_{em}$ = 260/336 nm, respectively. For LF, the fluorescence intensity of peak 1 also decreased from 483.59 to 228.56 and 129.17 due to the formation of the EC-LF complex and EGC-LF complex. The above results indicated that the interaction between catechin and LF changed the hydrophobicity of the LF microenvironment and LF conformation.

3.8. Molecular Docking Analysis of the Interaction between LF and EC/EGC. Molecular docking was adopted to acquire the binding sites of LF binding to EC and EGC. The lowest binding energies of the LF-EC and LF-EGC complexes were −8.472 and −8.644 kcal/mol, respectively, suggesting that both EC and EGC could bind stably with LF. Figure 5 displays that the hydrogen bond is both involved in the interaction between LF and EC/EGC. Table 4 shows the distance between EC/EGC and the amino acid residues of LF. The hydrogen atom of hydroxyl in EC formed one hydrogen bond with the oxygen atom of carboxyl group in Asp297 with the distance of 4.58 Å and formed one hydrogen bond with the oxygen atom of

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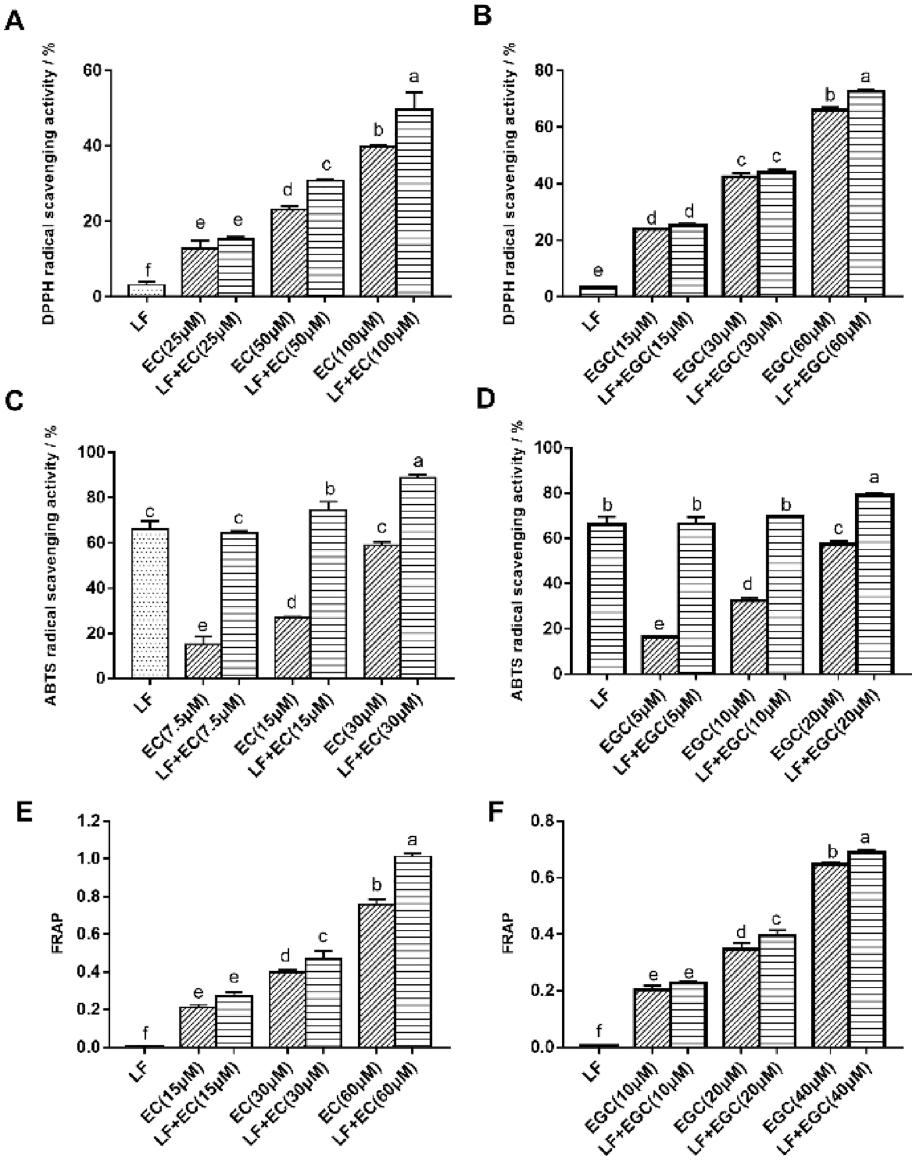

Figure 6. Antioxidant activity of LF, catechin, and catechin-LF complexes. (A) DPPH radical scavenging activity of LF, EC and LF-EC complexes. (B) DPPH radical scavenging activity of LF, EGC, and LF-EGC complexes. (C) ABTS radical scavenging activity of LF, EC, and LF-EC complexes. (D) ABTS radical scavenging activity of LF, EGC, and LF-EGC complexes. (E) FRAP of LF, EC, and LF-EC complexes. (F) FRAP of LF, EGC, and LF-EGC complexes. Different letters indicate significant differences between samples ($P \leq 0.05$).

carboxyl group in Glu15 with the distance of 5.71 Å. The oxygen atom of hydroxyl in EGC formed one hydrogen bond with the hydrogen atom of amino groups in Gly194, while the hydrogen atom of amino groups in His253 formed hydrogen bonds with the oxygen atom of hydroxyl in EGC with the distance of 4.79 and 4.29 Å. Moreover, the hydrogen atom of hydroxyl in EGC formed hydrogen bonds with the oxygen atom of carboxyl group in Thr58 with the distance of 2.94 and 4.63 Å (Table 4). EC formed π-cation with the amino acid residue of Arg121 with the distance of 6.29 Å and interacted with Phe190 via $\pi-\pi$ with the distance of 5.25 Å. EGC formed π-alkyl with Arg296 (7.59 Å) and $\pi-\pi$ with Phe190 (4.71 Å). Moreover, EGC interacted with Leu59 via the carbon hydrogen bond with the distance of 4.63 Å. Hydrogen bonds were also reported in the interaction between LF and dihydromyricetin and myricetin.[23] Due to the discrepancy in the hydroxyl number, the number of hydrogen bonds formed between LF and EGC was higher than that between LF and EC.

3.9. Effect of EC and EGC on the Antioxidant Activity of LF.

3.9.1. DPPH Radical Scavenging Ability of LF, LF-EC, and LF-EGC Complexes. The DPPH radical scavenging ability of catechin-LF complexes and catechin is displayed in Figure 6. As shown in Figure 5A,B, when the LF concentration was kept constant, the DPPH radical scavenging ability of the EC-LF complex and EGC-LF complex enhanced as the EC and EGC concentration increased, showing a significant dose−effect relationship. EC (25−100 μM) and EGC (15−60 μM) increased the DPPH radical scavenging ability of LF by 12.00−46.47 and 22.01−69.42%, respectively, which might be attributed to the interaction between LF and catechin.[41] Shi et al. also reported the covalent interaction between SPI and chlorogenic acid enhanced the DPPH radical scavenging ability of SPI.[42] Compared with EC, EGC at lower levels could increase the higher DPPH radical scavenging ability of LF. Moreover, the DPPH radical scavenging ability of the EC-LF complex and EGC-LF complex was significantly higher than that of EC and

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EGC alone with higher concentration. These results suggested that the EC-LF complex and EGC-LF complex with high ratios of catechin could simultaneously enhance the antioxidant activity of catechin and LF.

3.9.2. ABTS Radical Scavenging Activity of LF, LF-EC, and LF-EGC Complexes. Figure 6C,D displays the ABTS radical scavenging activity of the EC-LF complex and EGC-LF complex with different ratios of LF/catechin. LF alone had high ABTS radical scavenging activity of 66.01%, which was equal to that of the EC-LF complex with 7.5 μM EC and EGC-LF complex with 5 and 10 μM EGC. EC (30 μM) and EGC (20 μM) significantly increased the ABTS radical scavenging activity of LF by 22.69 and 13.17%, respectively. The higher antioxidant activity of protein-polyphenol complexes was related with the introduction of more hydroxyl groups on polyphenols.[35] EC and EGC had been reported to enhance the ABTS radical scavenging activity of SPI via modifying SPI.[43] Han et al. also reported that the ABTS radical scavenging activity of WPI-EGCG complex was higher than that of WPI alone.[44] It can also be seen that the ABTS radical scavenging activity of the EC-LF complex and EGC-LF complex was higher than that of EC and EGC alone at different concentrations. This finding also suggested that the innate antioxidant activity of LF enhanced the ABTS radical scavenging activities of EC and EGC.

3.9.3. FRAP Activity of LF, LF-EC, and LF-EGC Complexes. The FRAP activity of the EC-LF complex and EGC-LF complex with different ratios of LF/catechin is shown in Figure 6 E,F. The FRAP activity of the EC-LF complex and EGC-LF complex increased with the rising concentration of EC and EGC, suggesting a concentration−effect relationship. LF alone had the lowest FRAP value, and the FRAP activity of LF was remarkably enhanced by the different addition level of EC and EGC. EC (15−60 μM) increased the FRAP value of LF by 64.02−239.07 times, and EGC (10−40 μM) increased the FRAP activity of LF by 54.04−163.81 times. The results were in agreement with the FRAP activity of β-LG modified with EGCG[45] and laccase-catalyzed SPI-gallic acid complexation.[46]

The FRAP activity of EC-LF complexes with EC concentrations of 30 and 60 μM was higher than that of EC alone. EGC-LF complexes with 20 and 40 μM EGC also displayed stronger FRAP activity than that of EGC alone, which were in accordance with the DPPH radical scavenging ability and the ABTS radical scavenging ability of catechin-LF complexes increased by EC and EGC. Therefore, constructing catechin-LF complexes could be used as an effective strategy to enhance the antioxidant activity of LF and catechin.

4. CONCLUSIONS

This study explored the underlying mechanism of noncovalent interaction between LF and catechin and investigated the antioxidant activity of EC-LF complexes and EGC-LF complexes. Both EC and EGC quenched the fluorescence of LF in a static type and changed the microenvironment of tyrosine and tryptophan residues around LF. The binding forces between EC and LF depended on hydrophobic interactions, while hydrogen bonding and van der Waals forces drove the binding of EGC to LF. The key amino acid residues of LF binding to EC were different from that of LF binding to EGC, indicating that the discrepancy on ligand structure could affect the interaction between LF and EC/EGC. EC and EGC at higher concentrations could increase the antioxidant activity of LF. Meanwhile, the antioxidant activity of EC and EGC also could be increased by the formation of EC-LF complexes and EGC-LF complexes. This study will provide references for the application of LF−catechin complexes in the functional food industry.

■ ASSOCIATED CONTENT

Data Availability Statement

Due to all the data included in the manuscript, no supporting data has been supplemented.

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Author Contributions

Conceptualization was done by J.C. and S.M.; methodology was developed by X.C., X.D., and L.Z.; validation was performed by Y.Y., L.L., and H.Z.; writing—original draft preparation was done by J.C., G.L., G.R., and Q.X.; writing—review and editing was performed by W.C. and X.D.; funding acquisition was done by J.C., X.D., and G.R. All authors have read and agreed to the published version of the manuscript.

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Notes

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